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Extraction, Isolation, Characterization and DNA Binding Study of Carbazole Alkaloids from *Murraya Koenigii* (Curry Leaf)

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Abstract

Murraya Koenigii is a medium sized tree belonging to the family rutaceae. The present study aimed to investigate the phytochemical composition of the stem bark of murraya koenigii. The powdered plant material was extracted by maceration with acetone, and the acetone extract was fractionated with petroleum ether and subjected to column chromatography. The structure were established by interpretation of their UV-VIS, ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY NMR Spectroscopy and Mass spectrometry. The investigations led to isolation of two constituents were identified, such as Girinimbilol compound can be isolated by petroleum ether, another one compound cannot be isolated by column chromatography. The isolated compound was subjected to DNA binding study with Calf thymus DNA. The binding affinity value is 1.324 x 10^{5} m^{-1}. DNA binding study of the compound isolated from M.Koenigii is the first time reported method. The anti cancer study of the compound in the HeLa cell line with MTT assay method to IC_{50} value is 178 μM.

Keywords: *Calf thymus DNA; Chromatography; Girinimbilol; Murraya Koenigii.*

1. INTRODUCTION

Phytochemistry has developed in recent years as a distinct discipline. Somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structure of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function (Harbone, 1973). In this article we have studied about the curry leaf tree. It is a tropical to sub-tropical tree in the family *Rutacea*, which is native in india. The name itself in tamil is pronounced 'kariveppilai' and Malayalam 'kariveppila'. Literally, 'kari' means "curry", "veppu" and ilai/ila' leaf'. In kannada the name means "black neem" (Chatterjee and Pakrashi, 1997; Anonymous, 1997; Purthi, 1976). Since the appearance of the leaves are similar to the unrelated bitter neem tree. In the medicinal important of curry leaf plant has given many biological effects (Reisch *et al.* 1994). The medicinal organic compound is carbazole alkaloids represent a new and interesting variant in the number of exiting indole alkaloids which in the past yielded several important drugs (Narasimhan *et al.* 1968; Reisch *et al.* 1992; Kureel *et al.* 1970; Sharavanabhavan *et al.* 2013; Sampath and Jayabalakrishnan, 2013). A rich and revarding source of carbazole alkaloids has been the Indian curry leaf plant of murraya koenigii. All parts of this plant including root, stem, leaves and fruits yielded carbozole alkaloids. In views of the presence of various phytochemical constituents and pharmacological properties of this plant murraya koenigii. It was decided

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to work on the stem of murraya koenigii, which is widely used in indigenous medicine (Tachibana *et al.* 2001; Kureel *et al.* 1969; Narasimhan and Kelkar, 1976; Nayak *et al.* 2010). In the present investigation, we have used powder of curry leaf stem for extraction, isolated, characterization (UV-VIS, FTIR, 1D & 2D NMR and mass spectrometry) and biological effects of DNA binding study with Calf thymus DNA, and Anti cancer study on HeLa cell line.

1.1 Characterization

Melting points (M.P) of the extracted compound were determination in open capillary method on Raaga melting point apparatus and were uncorrected. The purity of the compounds was routinely checked by thin layer chromatography (TLC) using silica gel G as stationary phases. The instruments used for spectroscopic date are SYSTRONICS double beam UV-Vis Spectrophotometer: 2202, using standard reference methods. IR: PerkinElmer model RX1 (KBr) pellet method. NMR; Bruker 500 MHz spectrometer in $CDCl_3$, TMS (tetra methyl silane) as an internal standard references. DNA Binding study was carried out with SYSTRONICS double beam UV-VIS Spectrophotometer: 2202.

2. EXPERIMENTAL METHODS

2.1 Material and methods

The plant Murraya koenigii stem barks were collected freshly from Karamadai, Coimbatore district, Tamil Nadu, India in July 2011. All chemicals were used laboratory grade, petroleum ether (60-80 ^{0}C), chloroform, ethyl acetate, acetone, silica gel for TLC, silica gel (100-200 mesh) for Column and ethanol for extraction. Except ethanol all other solvents were purchased from Merck chemicals private Ltd. (India).

2.2 Preparation of Extraction

The shade dried powder of the stem bark (500 g) were placed into the extractor of a Soxhlet apparatus and subjected to extraction separately by hot percolation method. The extraction was carried out by using solvents of increasing polarity starting from petroleum ether (60-80 ^{0}C.), chloroform and acetone. The extraction was carried out with 250 millilitres of each solvent for a period of 6 hours. At the end of the extraction the respective solvents were concentrated by evaporation. These crude extracts were redissolved in respective solvents and the following experiments were carried out to stablish the presence of various constituents like alkaloids, sterols, coumarins, glycosides etc. The constituents were isolated from the crude extracts of petroleum ether, chloroform, acetone and ethanol.

2.3 Identification of the compound by using chromatographic method

After screening the various extracts obtained from 500g of bark powder, the petroleum ether was extract a dark green residue (2 gm). The chloroform extract was dark brown residue (3 gm). The acetone extract was dark brown residue (5 gm). The mobile phases were the increasing order of the polarity (petroleum ether < chloroform < acetone < ethyl alcohol < ethyl acetate). The petroleum ether, chloroform contains many-spot. But acetone extraction contained only two spots in petroleum ether mobile phase. Then the first spot gave two different spots in ethyl acetate mobile phase. These capillary actions were visualized by an iodine chamber.

The dark brown residue of acetone extract (5 gm) was poured into a column chromatograph packed with the silica gel (100-200 mesh). The two bands were separated with the solvent petroleum ether isolated as (band- I, band - II). The compounds A & B were isolated from band I - II respectively by using the solvent by pet.ether. The pure first compound was dried over a water bath. The compound is yellowish brown in colour. Another one compound B cannot be separate by column chromatography. The melting point of the pure compound was found to be 171-172 ^{0} C. The percentage of yield was 0.10%.

2.4. DNA-binding experiments

The UV-A at 260 nm and 280 nm of the Calf-thymus DNA (CT-DNA) solution in 5 mM Tris-HCl, 50 mM NaCl buffer (p^H-7.2) at room temperature gave a ratio of 1.8-1.9:1, indicating that the DNA was sufficiently free of protein. The concentration of CT-DNA per nucleotide was determined from its absorption intensity at 260 nm with a molar extinction coefficient of 6600 $M^{-1}cm^{-1}$ (Marmur and Doty, 1961; Reichmann *et al.* 1954). Stock solutions were stored at 4 ^{0}C, and used within 3 days. Titration experiments were carried out by varying the concentration of CT-DNA while keeping the isolated compound concentration constant (10 µm). The mixture was allowed to equilibrate for 5 min before spectra was recorded.

2.5. Cell treatment procedure

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). All cells were maintained at 37 ^{0}C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. The monolayer cells were detached with trypsin-ethylene diaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 ^{0}C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the extracts and fractions. They were initially dissolved in neat dimethylsulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 100, 50, 25, 12.5 and 6.25 µg/ml. The final volume in each well was 200 µl and the plates were incubated at 37 ^{0}C, 5% CO_2, 95% air and 100% relative humidity for 48 hr. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

2.6. Assessment of cell viability by MTT

The isolated compound (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hr of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ^{0}C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

3. RESULT & DISCUSSION

The TLC of the isolated compound showed a single spot in the polar solvent mixture pet.ether- ethyl acetate(2:1) which indicates that the compound is pure and polar. The UV-VIS absorption spectrum to give a peak at **369 nm** Fig. 1 indicates that the compound may contain an aromatic ring and double bond(s). In the visible region peak of **657 nm** indicates that the compound is yellowish brown colour.

The FT-IR spectrum of the compound is shown in Fig. 2. IR data reveals that the compound consists of aromatic ring, double bond and functional group. The N-H asymmetric stretching vibration appeared at 3369 cm^{-1}. The aromatic C-H asymmetric & symmetric stretching vibration appeared 2849 to 2917 cm^{-1} and C=C stretching of CH_2 group (olefinic) peak appeared at 1613 cm^{-1}. The spectral data are given in Table 1.

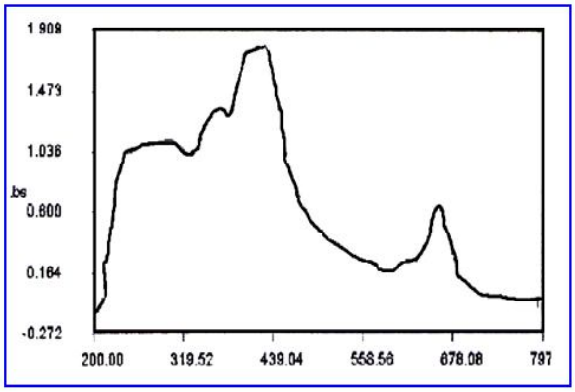

Fig. 1: UV-VIS Spectrum

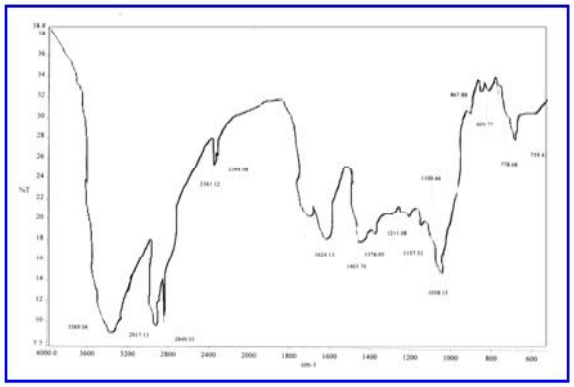

Fig. 2: FT-IR Spectrum

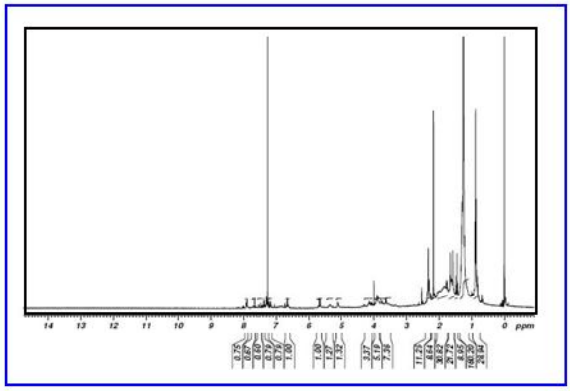

Fig. 3: ^{1}H NMR Spectrum

Table1. FT-IR Spectral data

Wave number (cm^{-1})	Assignments
3369	N-H Asymmetric stretching vibration
2917	Aromatic C-H asymmetric stretching vibration
2849	Aromatic C-H symmetric stretching vibration
1624	Aromatic conjugated stretching vibration
1463	C-O stretching vibration
1058	C-N stretching vibration
857 & 717	C-H out-of plane bending vibration

Table 2. The ^{13}C-NMR Spectral data of the compound

δ (ppm)	Assignment
14.12	C-10
22.69	C-12
29.68	C-13
29.36	C-9
31.94	C-11
107.61	C-2
110.92	C-7
112.51	C-4
119.01	C-6
120.22	C-5
123.48	C-4a
124.31	C-5a
125.39	C-8
127.90	C-1a
129.30	C-8a
139.41	C-1
145.30	C-12
76.5-77.5	Solvent

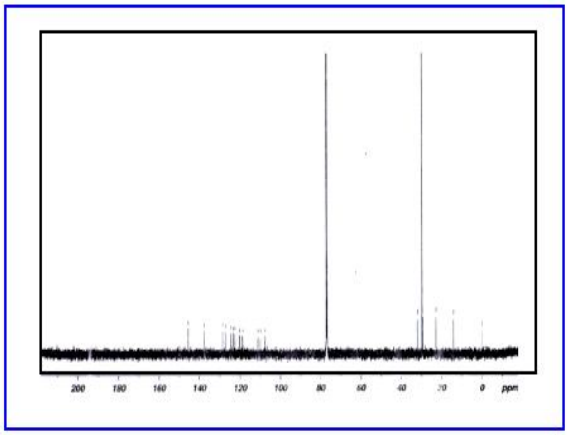

Fig. 4: ^{13}C NMR Spectrum

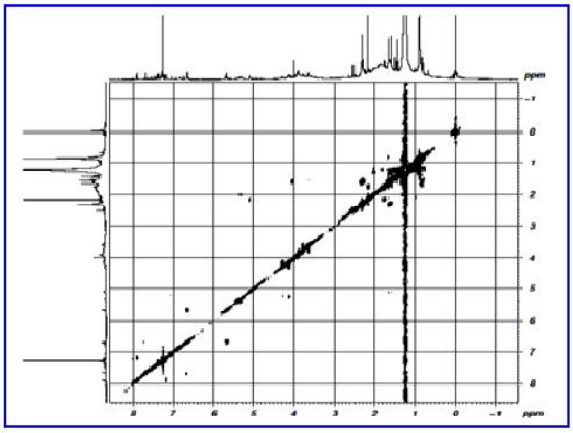

Fig. 5: ^{1}H-^{1}H COSY NMR Spectrum

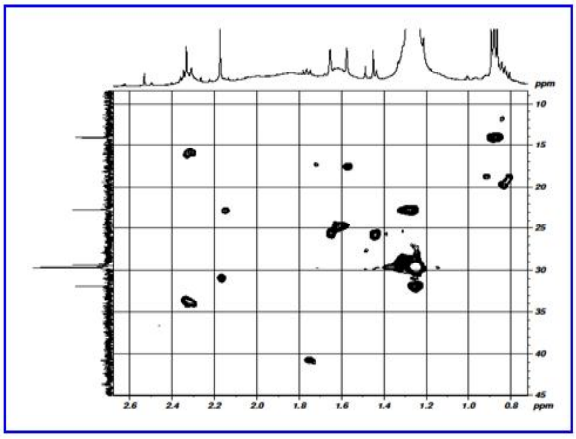

Fig. 6: ^{13}C-^{1}H COSY NMR Spectrum

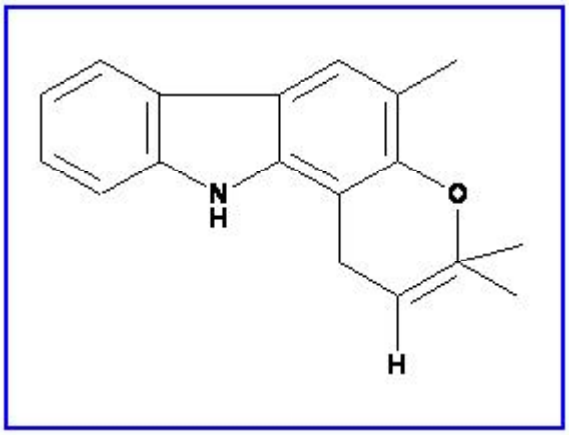

Fig. 7: Isolated compound of Girinimbilol

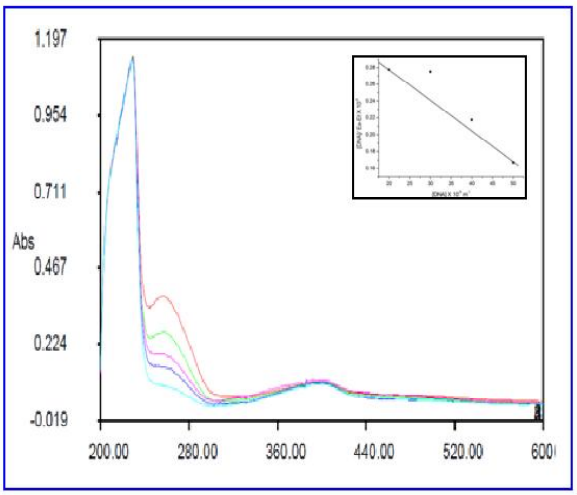

Fig. 8: DNA Binding spectrum: Electronic absorption spectra of isolated compound (25 mM) in the absence andpresenceof increasing amounts of CT-DNA (10, 20, 30, 40 and 50 mM). Arrows show the changes in absorbance with respect to an increase in the DNA concentration . (Insert: Plot of [DNA] vs [DNA]/(e_a-e_f)).

The ^{1}H-NMR spectrum was recorded in 500 MHz spectrometer Fig. 3. The NMR results further confirm the purity of the compound. The ^{1}H-NMR spectrum of the isolated compound shows eleven different proton environments. In the ^{1}H-NMR spectrum a doublet at δ 0.8 ppm corresponding to C-11 methyl proton. The splitting is due to four methylene proton present on two adjacent carbon atoms (C2-C4). The methyl group of whole system δ1.43 to 1.83 ppm. The C-3 proton appeared as quintet centred at δ 2.33 (J=6 Hz ; J=12 Hz). N-H protons are commonly strongly shielded, thus appearing in the spectral region of δ 1-3 ppm. The N-H proton appeared as a broad singlet at δ 2.53 ppm. The aromatic proton appearing at δ 6.64 to 7.91 ppm in the spectrum.

In the ^{13}C NMR spectrum Fig. 4 of carbon atoms appear between 24 and 145 ppm, corresponding to only aromatic and unsaturated carbon atoms. The peaks in the range 0 to 50 ppm, are due to a carbon-carbon single bond. The peaks between 100 and 150 are due to the aromatic carbon-carbon double bond assignment given in table 2. The DEPT NMR spectrum is helpful to identified by odd carbon and even carbon of the isolated compound. In the 2D NMR (^{1}H-^{1}H COSY & ^{13}C-^{1}H COSY) spectrum gives correlation of proton and carbon figs. 5 & 6. The 13 C spectral data are one used as a supporting evidence for the structure elucidation Girinimbilol fig.1.

4. DNA BINDING STUDY

Electronic absorption spectroscopy is one of the most useful techniques for DNA- binding studies of isolated compound. The interaction of isolated compound with CT-DNA was investigated by UV absorption titrations. The binding of the isolated compound to DNA helices was characterized by following the changes in the absorbance and shift in wavelength on each addition of DNA solution to the compound. A representative absorption spectrum is given in Fig. 8. The isolated compound in DMSO-buffer mixture exhibit an intense transition in the region around 310-225 nm, which is attributed to a π - π* transition that unique to this nitrogen base. On the titration of CT- DNA with the compound, a considerable increase or decrease in the absorption along with small blue shift was observed. With increasing concentration of DNA, the absorption bands of the compound were affected to a considerable extent. For compound, the absorption spectra show clearly that the addition of DNA to the compound yields hyperchromism and a blue shift to the ratio of [DNA]/[M]. Obviously, these spectral characteristics suggest that the entire compound interact with DNA most likely through a mode that involves a stacking interaction between the aromatic chromophore and the base pairs of DNA. Addition of increasing amounts of DNA resulted in hypsochromism of the peak maximam in the UV–VIS spectra of the compound. In the plot of [DNA]/(ε_a---ε_f) vs. [DNA], the binding constant Kb is given by the ratio of the slope to the intercept. The isolated compound having a binding constant value is 1.324 x 10^5 m^{-1}(Marmur and Doty, 1961; Reichmann *et al.* 1954).. In this value are given good binding of calf thymus DNA with isolated compound.

5. CYTOTOXICITY

To test the cytotoxicity of isolated compound, human cervical cancer cell line (HeLa) was cultured in

the presence of varying concentrations of compound for 48 h. The inhibitory concentration 50 (IC_{50}), defined as the concentration required to reduce the size of the cell population by 50%. The IC_{50} values of the compound (IC_{50} = 178 µM) exhibit enhanced activity against HeLa cell line. In this isolated compound show significant activity against the tumor cell lines. Thus, the IC_{50} value for the compound decreases with the natural compound which shows the more toxicity. However, these values are lower than the standard anticancer drug of cisplatin.

6. CONCLUSION

he preliminary phytochemical studies identified with the presence of carbazole alkaloids from acetone extract. Whereas, further phytochemical investication led to isolation of pure compound from petroleum ether fraction of acetone extract of stem bark of *Murraya Koenigii* the isolated compound was identified as *Girinimbilol*. This is one of the carbazole alkaloids compound. The structure of the compound was conformed with spectroscopic technique and biological effects of DNA binding study carried out with Calf thymus DNA, and anti cancer study were tested on HeLa cell line to MTT assay method.

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